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Research Article

Screening of *In-vivo* Anti-proliferative Activity of *Limonia acidissima* Against MCF-7 Cell Line

Tripathy Gitanjali*, Pradhan Debasish

University Department of Pharmaceutical Sciences, Utkal University, Vanivihar, Bhubaneswar-751004, Odisha, India.

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ABSTRACT

The aim of the study is to evaluate the anti-proliferative activity of *Limonia acidissima*. Anti-proliferative activity of methanolic extracts (200 and 400 mg/kg) of *Limonia acidissima* roots were evaluated against MCF-7 cell line in rats. Acute and short-term toxicity studies were performed initially in order to ascertain the safety of methanol extract of *Limonia acidissima* (MELA). After 24 h of tumor inoculation, the extract was administered daily for 14 days. After administration of the last dose followed by 18 h fasting, mice were then sacrificed for observation of anti-proliferative activity. The effect of MELA on the growth of transplantable murine tumor, life span of MCF-7 bearing hosts and simultaneous alterations in the hematological profile were estimated. The MELA showed decrease in tumor volume, packed cell volume and viable cell count, and increased the nonviable cell count and mean survival time thereby increasing life span of MCF-7 tumor bearing mice. Hematological profile reverted to more or less normal levels in extract treated mice. The methanolic extract of *Limonia acidissima* fruit pulp exhibited anti-proliferative activity in MCF-7 tumor bearing rat.

Key words: *Limonia acidissima*; Anti-proliferative agent; MCF-7

INTRODUCTION

Cancer is the second leading cause of death in the world[1]. Currently over 60 % of used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development[2,3]. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine. *Limonia acidissima* is a commonly occurring tropical plant species belonging to the Family Rutaceae. It is native of India, China, Indonesia and Sri Lanka. Its vernacular names are wood apple, elephant apple, monkey fruit, curd fruit and kaith bel[4]. In tribal areas of western Odisha the plant is popularly known as *Kaintha*. The literature survey of traditional herbal cures reveals that *Limonia acidissima* was used as a medicinal plant in ancient Greek and Roman times and in 'Ayurveda' as a vasodilator, antimicrobial, laxative, purgative, astringent and antihypertensive herb. Fruit pulp and rind of this plant are extensively used against snake-bite and stings of venomous insects[5]. The fruits of the plant are edible and generally considered to be stomachic, astringent, diuretic, cardio-tonic and tonic to liver and lungs[6]. The unripe fruit is used to treat diarrhoea and dysentery and is also an effective treatment for cough, sore throat and diseases of the gums.

Leaves are aromatic and carminative and are used for the treatment of indigestion and minor bowel infections in children[7]. Antibacterial activity of the leaf essential oil and stem bark has been already reported[8,9] but no work has been published on the antimicrobial potential of leaf, fruit pulp and fruit rind extracts of this plant. The anticancer activity of fruit pulp of *Limonia acidissima* had been previously reported against SKBR3 and MDA-MB 435[10]. The ripe fruit pulp is also reported to possess immunomodulatory activity[11].

Major classes of anticancer compounds include alkaloids, terpenoids, flavonoids, and lignans[12]. Plant derived natural products such as flavonoids, terpenoids, and steroids *etc* have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antiproliferative activity[13]. The present study was carried out to evaluate the antiproliferative activity methanolic extract of *Limonia acidissima* (MELA) against MCF-7 in Wister albino rats.

MATERIALS AND METHODS

Collection of plant material and extraction

Ripe fruits of *Limonia acidissima* was collected from the forest part of Bhubaneswar hill area situated in the eastern part of India in the month of May and identified by Dr. S K Sahu, a taxonomist at Utkal University, Vanivihar, Odisha, and Dr. S P Panda, Herbarium keeper, Regional Plant Research Center, Bhubaneswar. Voucher specimens were deposited in the herbarium of the Department of Botany, Utkal University. The fruits were cut into small pieces and the pulp was separated from the seeds. The pulp was shade-dried, and milled. The coarsely powdered, shade dried fruit pulp of both the plants was first defatted with Petroleum ether using soxhlet apparatus.

**Author for Correspondence*

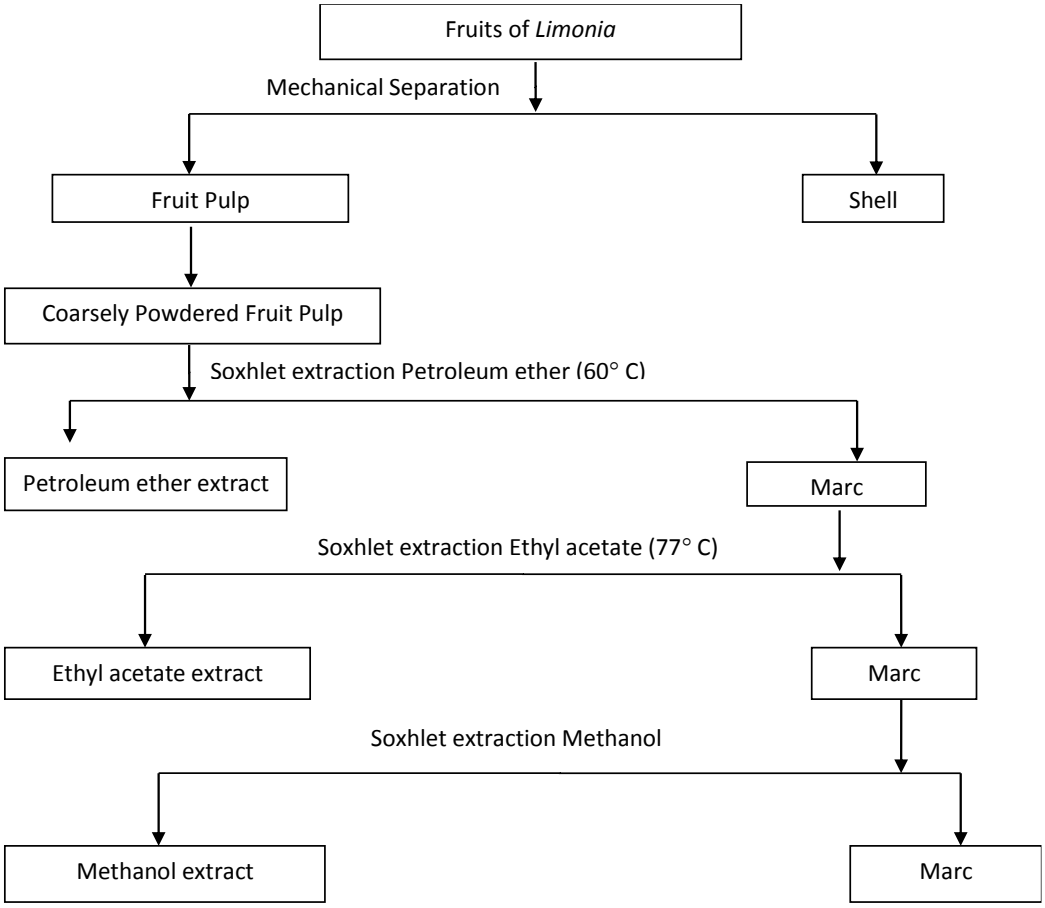

Fig.1: Scheme of extraction for the Fruit pulp of *Limonia acidissima*

The extracts were concentrated using rotary evaporator to get solid residue. The marc from the central compartment was removed, dried and extracted by exhaustive extraction with a series of solvents of increasing polarity with Soxhlet extractor was done[14]. The weight of the residue extracts were recorded and percent yield calculated. Solvents used with increasing polarity are petroleum ether, ethyl acetate, methanol and the scheme of extraction was depicted as a flow chart (Figure 1) below.

Preliminary Phytochemical Screening

The percentage yield of other extracts except methanolic extract was negligible. So,the methanolic extract was taken for further experimental work. The prepared methanolic extract was subjected to routine phytochemical analysis[15] to identify the presence of various phytochemicals such as carbohydrates, alkaloids, glycosides, saponins, flavonoids, tannins, sterols, phenols, etc.

Cell Culture

Human breast cancer MCF-7 cell line was obtained from Sigma-Aldrich, Bangalore. MCF-7 is a cell line used in many studies due to its characteristics and being easy to culture. The MCF-7 cell line is adherent and grows in clumps. The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Experimental animals

Adult albino female rats of Wistar strain weighing 180 - 240 g were used in the anti-breast cancer studies. They were housed in a controlled environment (temperature 25±2^{0}C and 12 hr dark/light cycle) with standard laboratory diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into six groups of each six animals. They were kept in standard polypropylene rat cages with stainless steel top grill supplied by Inco, India, with sterilized paddy husk as the bedding material. The animals were fed with balanced rodent pellet diet and tap water was supplied throughout the experimental period. The drinking water and the feed were provided *ad libitum*, except during fasting. Prior to the study, the experimental protocol was submitted to the CPCSEA/ IAEC and got approved from Pinnacle Biomedical Research Institute, Bhopal and the Approval number being 1283/c/09/CPCSEA.

Methods

Acute oral toxicity study

Acute oral toxicity test was performed by following OECD guideline–420[16]. Increasing doses of the extracts, up to 2000 mg/kg body wt, were administered to the rats.

Anti Breast Cancer Activity

After acclimatization, mature female wister albino rats divided into four groups (n=6) and given food and water *ad libitum*. The entire groups except group I were injected with MCF-7 cells (2×10^6cells/rat.i.p). This was taken as day 0. The animals were grouped as follows (n=6): Group I: The animals of this group received normal saline (5 ml/kg, p.o); Group II: The animals of this group were

Table 1: Effect of MELA on haematological parameters and body weight of treated rat.

Parameters		Vehicle control 5 ml/kg	MCF-7 (2 x 10^6 cell)	MCF-7 (2 x 10^6 cell) + MELA (200mg/kg p.o)	MCF-7(2 x 10^6 cell) + MELA(400 mg/kg p.o)	MCF-7 (2 x 10^6 cell) + Doxorubicin (20 mg/kg)
Hb/g %		11.60±0.12	5.12±0.13[a]	9.30±0.16	11.06±0.25[a]	11.34±0.20
RBC/10^6/mm^3		6.60±0.20	3.7± 0.1[a]	5.8±0.35	6.2±0.4	6.4±0.13[a]
Total WBC/10^3/mm^3		5.40±0.19	11.2±1.2 [a]	10.90±0.22	6.30±0.21	6.75± 1.5 [a]
Total Protein (g/%)		8.5±0.2	14.6± 1.5 [a]	11.2±0.7 [a]	8.6±0.7 [a]	8.4±2.1 [a]
Differential count	Lymphocytes	70.2±1.31	30.3±0.4 [a]	55.8±1.1 [a]	67.9±2.1[a]	68.7±1.1 [a]
	Neutrophil	29.8±1.1	68.6±1.6 [a]	35.6±1.6 [a]	32.1±1.3 [a]	30.4±1.6 [a]
	Monocytes	2.2±0.4	3.8±0.5[a]	2.9±0.4	2.8±0.3	2.1±0.4
Body weight/g		181.70±0.19	228.12±0.35[a]	168.10±0.13	181.30±0.15	181.50±0.5[a]

n=6, Mean ± SEM, [a]P<0.01*vs.*MCF-7 control group

Table 2: Effect of MELA on mean survival time, % ILS, tumor volume, packed cell volume, viable and non-viable tumor cell count

Parameters	Vehicle control 5 ml/kg	MCF-7 (2 x 10^6 cell)	MCF-7 (2 x 10^6 cell) + MELA (200mg/kg p.o)	MCF-7 (2 x 10^6 cell) + MELA (400mg/kg p.o)	MCF-7 (2 x 10^6 cell) + Doxorubicin
Mean survival time /d	48.12±0.13[c]	24.00±0.11	32.14±0.16[c]	44.15±0.19[c]	47.85±0.15[c]
Increase life Span/%	100	–	33.33	83.33	99.37
Tumor volume/ml	–	4.51±0.07	2.41±0.03[c]	1.20±0.01[c]	–
Packed cell volume/ml	–	2.11±0.84	0.81±0.01[c]	0.23±0.01[c]	–
Viable tumor cell count/10^{10}cells/l	–	12.30±0.07	8.84±0.06	0.68±0.04[c]	5.04±0.04[c]
Non Viable tumor cell count/10^{10}cells/l	–	0.89±0.06	1.57±0.05[c]	-	-

n=6. Mean±SEM.[c]P<0.01 *vs* MCF-7 control group.

injected with MCF-7 cells only without any drug treatment (2×10^6 cells/rat. i.p); Group III: Received a MELA at a dose of 200 mg/kg body weight dose administered orally for 14 consecutive days + MCF-7 Cells (2×10^6 cells/rat. i.p); Group IV : Received a MELA at a dose of 400 mg/kg body weight dose administered orally for 14 consecutive days + MCF-7 Cells (2×10^6 cells/rat. i.p); Group V : Received a Doxorubicin (standard drug) at a dose of 20 mg/kg body weight dose administered orally for 14 consecutive days + MCF-7 Cells (2×10^6 cells/rat. i.p).

On day 1, MELA at a dose of 200 and 400 mg/kg body weight (Gr-III and IV) were administered orally for 14 consecutive days. Gr V was administered with Doxorubicin 20 mg/kg b.wt. orally for 14 days. On day 15, three rats from each group were sacrificed 24 h after the last dose and the rest were kept with food and water *ad libitum* to check the life span of the tumour hosts[17].

The effect of test groups on tumour growth and host's survival time were examined by studying the parameters like tumour volume, tumour cell count, viable tumour cell counts, nonviable tumour cell count, mean survival time, packed cell volume (PCV)[17] and increase in life span (% ILS)[18,19].

An enhancement of life span by 25% or more was considered as effective antitumour response[20, 21]. Recording the mortality monitored the effect of MELA on tumour growth[22] and percentage increase in life span (ILS %)were calculated (Hogland, 1982) by the following formulae:

$$\text{MST} = \frac{(Day\ of\ first\ death + Day\ of\ last\ death)}{2}$$

$$\text{ILS (\%)} = \left[\left(\frac{Mean\ survival\ time\ of\ treated\ group}{Mean\ survival\ time\ of\ control\ group}\right) - 1\right] \times 100$$

Hematological studies

Hemoglobin content, red blood cell (RBC), white blood cell (WBC) counts and total protein (biuret's method) were measured from freely flowing tail vein blood [23]. Differential WBC leukocyte count was carried out from Leishman stained blood smears of normal, MCF-7, and MELA treated groups respectively[24].

Statistical analysis

The experimental results were expressed as mean ± S.E.M. Data were assessed by the method of One-way ANOVA followed by Dunnett's posthoc test. P value of <0.05 was considered as statistically significant.

RESULTS

Acute toxicity studies

The extracts of *L. acidissima* did not provoke any gross behavioral changes or manifestations of toxic symptoms in the animals, such as weight loss, increased motor activity, tremors, spasticity, loss of right reflex, decreased motor activity, ataxia, sedation, muscle relaxation, hypnosis, arching and rolling, lacrimation, salivation, watery diarrheoa, writhing and urination, over an observation period of 24 h. The extracts were non-lethal even at the maximum single oral dose of 2000 mg/kg.

Effect of MELA on mean survival time and tumor growth

The average tumour volumes for MCF-7 treated animals were found to be 4.51±0.07. MELA treatment at both dose levels (200 and 400 mg/kg) significantly (P<0.01) reduced tumour volume which was found to be 2.41±0.03[c] and 1.20±0.01[c]respectively. Viable cell count of the tumour bearing rat (12.30±0.07) was significantly decreased i.e, (8.84±0.06) and (0.68±0.04[c]) in case of MELA 200 and 400 mg/kg respectively. While non- viable cell count also decreased i.e, (1.57±0.05[c]) in MELA 200 mg/kg and even no nonviable tumor cells were found in MELA 400 mg/kg group and Doxorubicin group.

The effect of MELA on the survival of tumour bearing rat on MST for MCF-7 treated group to be 24.00±0.11 days, while it was 32.14±0.16days (33.33%) and 44.45±0.19 days (83.33%) for the animals treated with MELA at the dose of 200 and 400 mg/kg respectively. Treatment with Doxorubicin enhanced survival rate to 47.85±0.15[c]days (99.37%) as compared to the control group.

Effect of MELA on hematological parameters

The haemoglobin content in cancer control group (5.12 ± 0.13[a]) was significantly (p<0.01) decreased as compared to the normal group (11.60 ± 0.12). Treatment with 200mg/kg MELA and 400mg/kg MELA significantly increases the haemoglobin content to more or less equal to normal levels i.e, (9.30 ± 0.16) and (11.06 ± 0.25) respectively. 400mg MELA treated group (p<0.01) had shown significant increase in haemoglobin content when compared to other dose of the extract treated groups. Treatment with Doxorubicin (20 mg/kg) increased the haemoglobin content to nearly normal levels i.e. (11.34 ± 0.20).

The RBC count in the cancer control group (3.7 ± 0.1[a]) was decreased as compared to the normal group (6.60 ± 0.20). Treatment with 200mg and 400mg/kg MELA increased the RBC count to normal levels i.e, (5.8±0.35) and (6.2±0.4[b]). Treatment with standard drug, Doxorubicin was shown to increase the RBC count (6.4±0.13[a]) when compared with Control group (6.60 ± 0.20).

The total WBC count was found to have increased significantly in the cancer control group (11.2±1.2[a]) when compared with normal group (5.40±0.19) (p <0.01). Administration of 200mg/kg and 400mg/kg MELA in MCF-7 bearing rat reduced the WBC count i.e, (10.90±0.22) and (6.30±0.21) respectively as compared with cancer control group. Also administration of 200 mg/kg MELA (P <0.01) and 400mg/kg MELA (p <0.01) in MCF-7 bearing rat significantly reduced the WBC count as compared with cancer control group. Treatment with Doxorubicin (20 mg/kg) decreased the WBC count i.e. (6.75 ± 1.5[a]) as compared to the MCF-7 group (11.2±1.2[a]). 400mg/kg MELA treated group had significantly reduced the WBC count when compared to the other dose of the extract treated groups.

The total protein was found to be increased significantly in the cancer control group (14.6±1.5[a]) when compared with the normal group (8.5 ± 0.2). Administration of 200 and 400 mg/kg MELA treated groups had shown a significant decrease in the total protein i.e, (11.2 ± 0.7[a]) and (8.6 ± 0.7[a]) respectively when compared to MCF-7 treated group (p<0.01). Treatment with Doxorubicin (20 mg/kg) decreased the total protein count i.e. (8.4 ± 2.1[a]) as compared to the MCF-7 group (14.6±1.5[a]).

In the differential count of WBC, number of neutrophils increased and lymphocytes decreased in MCF-7 group. The test extract groups altered these parameters more or less to normal.

The body weight of MELA treated 200 and 400 mg/kg groups was found to be decreased to (168.10±0.13) and (181.30±0.15) respectively as compared to MCF-7 treated group (228.12±0.35). The increase in weight may be due to the presence of tumor which was then decreased upon treatment of test extracts and the standard drug, Doxorubicin (20 mg/kg).

The detailed results are shown in Table 2.

DISCUSSION

The present study was carried out to evaluate the toxicity and in-vivo anti-breast cancer activity of MELA on MCF-7 bearing rats. The reliable criterion for judging the value of any anticancer drug is the prolongation of lifespan of the animal and decrease of WBC count from blood[25].The above results demonstrated the anti-breast cancer effect of MELA against MCF-7 in Wistar albino rats. A significant (P<0.05) enhancement of MST and non-viable cell count in peritoneal exudates (P<0.05) was observed due to MELA treatment.

To evaluate whether MELA treatment indirectly inhibits tumour cell growth, the effect of MELA treatment was examined on the viable and non-viable cell counts against tumour bearing rat. Normally, each rat contains about 2 x 10^6 intraperitoneal cells, 50% of which are macrophages. MELA treatment was found to decrease both viable and non-viable tumor cell counts in peritoneal exudates. It might be due to the absorption of MELA by viable cells which leads to lysis of cell through to the activation of macrophages or some cytokine production in peritoneal cavity.

The elevated WBC count in tumour bearing rat was significantly reduced by MELA treatment. The MELA treatment inhibits tumour cell growth, enhances the survival of treated rat and restores the haematological parameters. Thus our present study suggests that MELA possess potent anti breast cancer activity and increases the life span of the treated animal. However MELA (400 mg/kg) shows more significant anti breast cancer activity among all test extract groups. Usually, in cancer chemotherapy, the major problems that are being encountered are of myelosuppression and anemia, but the results have clearly shown that MELA treatment has not only brought back hemoglobin content to normal but also the RBC count to normal. MELA had also found to be immunomodulatory property[10] and anticancer activity against cell lines like SKBR3 and MDA-MB-435[11]. Analysis of the other haematological parameters showed minimum toxic effect in the rat which were treated with MELA. After 14 days of transplantation more significantly MELA treated groups were able to reverse the changes in the hematological parameters consequent to tumour inoculation.

The higher rate of reduction in solid tumor volume indicated that MELA plays a direct role in killing tumor cells and enhances the curative effect tumor chemotherapy. A decrease in tumor volume and viable tumor cell count as mentioned above reduced the tumor burden and might have enhanced the life span of the tumor bearing rats.

The preliminary phytochemical study results indicated the presence of flavonoids and terpenoids in MELA. Flavonoids have been shown to possess antimutagenic and antimalignant effects [26, 27]. Furthermore, flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation[28] and angiogenesis[29]. Thus, the antitumor effect produced by MELA may be due to the presence of flavonoids and terpenoids content as well as its antioxidant and immunomodulatory potential. Many mechanisms are responsible for the anti-breast cancer activity; most important of these is that the genes that control apoptosis have a major effect on malignancy through the disruption of the apoptotic process that leads to tumour initiation, progression and metastasis. Therefore, one mechanism of tumour suppression by natural products may be to induce apoptosis, thereby providing a genetic basis for cancer therapy by natural products. Another mechanism for the anti-breast cancer action of MELA and its constituents is the inhibitory effect on free radical chain reactions, because most limonoids might act as membrane-associated high efficiency free radical scavengers, connects with their antioxidant properties[30].

Ascitic fluid is the direct nutritional source to tumour growth could possibly be a makes to meet more nutritional requirement of tumour cells. A regular rapid increase in ascites tumour volume was noted in tumour bearing rat. Linear progression in the body weight of tumour bearing rat with advancement of duration was observed. Such increase in body weight was significantly seen following the MELA treatment. Most anticancer drugs are antiproliferative and will also affect radically dividing normal cells.

In cancer chemotherapy the major problem is of myelosuppression and anemia. The anemia encountered in tumor bearing rats is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with MELA extracts brought back the hemoglobin content, RBC and WBC cell count near to normal values.

Plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food[31]. Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified[32]. Phenolic compounds of plants are also very important because their hydroxyl groups confer scavenging ability. Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant and anticancer activities[33].

Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti inflammatory, anticancer, and anti-allergic activities[34, 35].

The methanolic extract of *Limonia acidissima* possess flavonoids as exhibited in the phytochemical screening. These flavonoids may be the major contributors for the anti-breast cancer activity which exhibited significant correlation. Both the extracts restored the mean survival time, decrease tumor volume, cancer cell count in treated rat. Thus our present study suggests that MELA 400 mg/kg possess significantly higher anti breast cancer against MCF-7 breast cancer cell line and increases the life span of the treated animals as compared to the other test groups.

CONCLUSION

All these data point to the possibility of developing methanolic extract of fruit pulp of *Limonia acidissima* as a novel, potential agent in the area of cancer chemotherapy. The present study highlighted the anti-breast cancer activity of MELA in *in vivo* experimental models against MCF-7 cells induced tumor and thereby proves the supportive traditional claim of the plant part. Further works are also carried out to isolate and identify the active principle involved in the anti-breast cancer activity of the methanolic extract of *Limonia acidissima* (MELA).

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